Rapid detection of Candidatus Liberibacter asiaticus, the bacterium associated with citrus Huanglongbing (Greening) disease using PCR

Citrus occupies an important place in the horticultural wealth and economy of India. Presently it is the third largest fruit industry after mango and banana. Huanglongbing (HLB) (Greening) is one of the most serious diseases of citrus, occurring in African and Asian countries, including India. The disease causes substantial economic losses to the citrus industry by shortening the life span of infected trees. In endemic orchards, trees are decimated and the productive duration of fruit-bearing is reduced. The etiologic agent of the disease is a Gram-negative bacterium which is restricted to the phloem sieve tubes of infected plants. Infection of plants occurs through two psyllid vectors, Diaphorina citri in Asia and Triozoa erytreae in Africa. Despite many attempts, the HLB bacterium has not yet been cultured. However, by studying the nucleotide sequence of its 16S ribosomal RNA gene (rDNA), the bacterium has been characterized as a new genus belonging to the alpha subdivision of the proteobacteria with the trivial name Candidatus Liberibacter. The designation Candidatus Liberibacter asiaticus was given to the Asian greening bacterium, according to the rules established for uncultured organisms. HLB is a serious problem affecting the production of citrus in many areas of India, especially in Maharashtra, where two most popular cultivars of citrus, e.g. Nagpur mandarin (Citrus reticulata) and Mosambi sweet orange (Citrus sinensis) are cultivated on a commercial scale. Successful management of the disease requires effective and rapid detection of the Liberibacter agent and monitoring the progress of epidemics. The detection technique followed for HLB in India is primarily dependent on biological assay on indicator hosts (biological indexing) which requires big spacing under controlled environmental conditions, availability of large number of good indicator hosts and a long time, generally 4–6 months, for symptom expression. Moreover, detection becomes difficult sometimes because of its low concentration and uneven distribution in its natural hosts. The sensitivity of HLB bacterium detection has recently been enhanced by the development of polymerase chain reaction (PCR) with adequate primer pairs. In this study PCR was used to detect the presence of Liberibacter in samples of citrus plants collected from various places in Maharashtra.

Survey trips were made to different localities of Maharashtra during Jan.–Feb. 2003. Buds were collected from trees with green tip symptoms. Shoots were side grafted to one-year-old sweet orange cv. Mosambi indicator plants. Three plants were inoculated from each source and maintained separately in an insect-proof screen-house. DNA was extracted from leaf midribs and bark of the indicator plants by cetyltrimethylammonium bromide (CTAB) procedure, six months after grafting. Leaf midribs and bark tissues (~500 mg) were powdered in liquid nitrogen, and each sample was suspended in 1.5 ml of DNA extraction buffer (100 mM Tris-HCl (pH 8.0), 100 mM EDTA, 250 mM NaCl, 1% N-lauroylsarcosine), and transferred to a 1.5 ml Eppendorf tube. After incubation at 55°C for 1 h, the sample was centrifuged at 6000 rpm for 5 min. The supernatant (800 μl) was collected, and 100 μl 5 M NaCl and 100 μl 10% CTAB in 0.7 M NaCl were added, and the mixture incubated at 65°C for 10 min. The sample was subjected to one cycle of chloro-
The presence of HLB disease symptoms as described earlier was noted at each locality surveyed. Infected leaves were small, upright and frequently had various types of chlorotic mottling and Zn-deficiency-like symptoms. Diseased trees were sparsely foliated, affected by extensive twig die-back (Figure 1). Typical symptoms of disease (severe vein yellowing and leaf mottle on the emerging shoots) on the inoculated indicator plants developed within 4–6 months after grafting. PCR using primers OI1 and OI2c produced an amplified fragment of expected size (1160 bp), which was observed in Mosambi indicator plant samples infected by various isolates of Candidatus L. asiaticus collected from various places in Maharashtra (Figure 2). No amplification was obtained from water or DNA extracted from healthy citrus. Compared to the more conventional biological indexing, this PCR method permitted much more rapid detection of HLB bacterium. The protocol basically consists of three major steps: (i) extraction of template DNA from citrus tissue; (ii) PCR amplification and (iii) analysis of PCR products. The entire procedure can be completed within a 6 h time period: 2 h for DNA extraction, 3 h for PCR, and 1 h for analysis of PCR products by agarose gel electrophoresis. The PCR-based assay was also able to overcome difficulties caused by the low concentration and uneven distribution of HLB bacterium in citrus hosts. This PCR-based molecular indexing technology offers tremendous scope to produce a larger number of HLB-free citrus nursery plants within a lesser time period in the budwood certification programme and should also be useful in managing and monitoring disease epidemics.


**Figure 1.** Huanglongbing (greening) disease symptoms in Mosambi sweet orange (Citrus sinensis) tree showing sparsely foliated branches and twig die-back. A healthy tree is seen in the background. (Inset) Small, chlorotic, HLB-infected leaves with Zn-deficiency-like mottling.

**Table 1.** Primer sequences used for PCR amplification of 16S rDNA of citrus HLB bacterium

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide sequence</th>
<th>Nucleotide position</th>
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<tbody>
<tr>
<td>OI1</td>
<td>5′-GGCGCGTATGCAATACGACGCA-3′</td>
<td>39–61*</td>
</tr>
<tr>
<td>OI2c</td>
<td>5′-GCCTCGGCAGCTTGCCAAACC-3′</td>
<td>1183–1204</td>
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*Nucleotide positions are numbered following sequences in the EMBL database L 22532.

**Figure 2.** Agarose gel electrophoresis of DNA amplified with primers OI1 and OI2c from leaf midrib and bark tissue extracts. Lane 1, Water; lane 2, Extract from healthy citrus; lanes 3–8, Extracts from HLB-infected citrus from different areas of Maharashtra: Roha (lane 3), Jalalkha (lane 4), Pathan, Aurangabad (lane 5), Wasiwadi, Ahmednagar (lane 6), Sainu (lane 7), Dewalburi, Jhuna (lane 8), M, 1 kb ladder.

Form/isooamyl alcohol (24:1) extraction, and the aqueous supernatant was then re-extracted by an additional cycle of phenol/chloroform/isooamyl alcohol (25:24:1). The nucleic acids were precipitated by mixing 600 μl of the supernatant and 360 μl (0.6 volume) isopropanol followed by centrifugation at 12,000 rpm for 30 min. The pellets were washed with 70% ethanol, dried, and resuspended in 100 μl TE (10 mM Tris-HCl (pH 8.0), 1 mM EDTA) buffer. PCR was conducted in 50 μl reaction mixtures [10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM MgCl2, 0.2 mM each dNTP, 2.5 units of Taq DNA polymerase (MBI Fermentas) and 0.2 μM each of primers (Operon)]. The primers used were designed on the basis of the sequence information reported, which is conserved among Asian strains of the Liberibacter. Primers – OI1 and OI2c defined from the 16S rDNA sequence of L. asiaticus (strain Poona, India) (Table 1) were designed. The PCR reaction was carried out in a Palm Cycler (Corbett Research, Australia) first by incubation at 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 56°C for 1 min, 72°C for 2 min and final extension of 10 min at 72°C. The amplified DNAs (PCR products) were analysed on 1.4% agarose gel stained with ethidium bromide.
Inducible expression of green fluorescence protein in *Lactococcus lactis*

*Lactococcus* are lactic acid bacteria widely used in various foods and fermentation processes. A great deal of interest has been shown to genetically modify these economically important organisms to improve their traits by introducing specific genes through cloning techniques. In addition, since the lactic acid bacteria are considered GRAS (generally regarded as safe) organisms, considerable interest exists in the development of genetic tools that allow production of important proteins in lactic acid bacteria.

Due to the lack of commercially available expression vectors for *Lactococcus*, it is important to construct expression vectors to enable the expression of genes of interest in them. Several available vectors such as pMG36e have a constitutive promoter and therefore do not allow for controlled expression of the recombinant proteins. An improved expression vector can be constructed by replacing the constitutive promoter with those that are inducible. The nisin-based promoter has been shown to be a useful promoter for expression of genes in *Lactococcus*.

Nisin-inducible gene expression system is based on the autoregulatory properties of the *L. lactis* nisin gene cluster. Nisin is a small, cationic, hydrophobic peptide of 32 amino acids that belongs to the lanthibiotic class of bacteriocins. Two genes in the cluster, *nisA* and *nisF*, are induced by nisin via a two-component signal transduction pathway containing a histidine protein kinase, NisK, and a response regulator, NisR. Expression of both *nisR* and *nisK* is driven by the constitutive promoter. Nisin acts as an inducer on the outside of the cell and is sensed by NisK. Recently, it has been reported that two-plasmid systems in which the *nisA* promoter and the regulatory genes *nisR* and *nisK* are used, allow efficient control of gene expression by nisin in a variety of lactic acid bacteria.

The green fluorescence protein (GFP) used in this study was isolated from the Pacific jellyfish, *Aequorea victoria*. GFP is a protein of 238 amino acids, which spontaneously emits green light at 508 nm when excited with blue light at 395 nm in the presence of O2. GFP has the advantage of being an auto-fluorescent protein that does not require a substrate. This fact allows its detection in living cells and in real time. Measurement of GFP activity requires a post-translational oxidation of the protein.

Here we constructed an inducible expression vector for *L. lactis* based on the *nisA* promoter and studied its functionality using GFP as the reporter protein. The inducibility of the promoter using galactose as the alternative inducing agent was also studied. This simple expression system would contribute to the pool of available expression systems for *Lactococcus* and could be utilized as a useful tool for studying the expression of genes of interest in *Lactococcus*. We also showed that GFP could be used as a reporter gene for analysis of promoters in *L. lactis*.

*Lactococcal* cells were grown at 30°C in M17 broth containing 0.5% glucose as standing culture. *Escherichia coli* cells were grown at 37°C with vigorous agitation in LB broth. Whenever required, a total concentration of 5 μg/ml of erythromycin was used for *Lactococcus*, while 150 μg/ml of erythromycin was used for *E. coli* cultures.

Molecular cloning techniques were performed essentially as described by Sambrook *et al.*. Restriction enzymes, T4 DNA ligase and deoxynucleotides, were used according to the instructions of the supplier. Electroporation of *L. lactis* were performed using a gene pulser. *E. coli* was used as an intermediate host for cloning and plasmid DNA was isolated from *E. coli* using the alkaline lysis method. DNA fragments were isolated and purified with agarose gel (Qiagen, USA) before being subjected to PCR. PCR was performed in 25 μl reaction volumes in the presence of 1X PCR buffer with 1.5 mM MgCl2 (Fermentas, USA), 2.5 U Pfu DNA polymerase (Fermentas, USA), 5 mM dNTP (Fermentas, USA), 0.4 μM of each primers, 1 μl (0.1 μg) of template (genomic DNA of *L. lactis* ATCC 11454). Samples were denatured at 95°C for 1 min, 60°C for 1 min, 72°C for 45 s, and after the last cycle the reaction was held at 72°C for 10 min. Genomic DNA of *L. lactis* strain ATCC 11454 was isolated using the method of Engelke *et al.*, with minor modifications. PCR technique was applied to amplify the promoter region of *nisA* gene. This is a 215 bp fragment from positions 156 to +3 with respect to the nisA promoter transcription start site, which includes the −35 and −10 sequences and the ribosomal binding site of *Lactococcus*. Primers were designed based on the published sequence of *nisA* gene.

The forward primer was used to carry EcoRI site and reverse primer was designed to carry XmaI site. PS1 is a 30-mer forward primer with a sequence of 5'-GAATTCCAGTTCTTAGACATACTC-3' and Vnis is a 27-mer reverse primer with a sequence of 5'-CCCCGGGTCACTCATTGTTGACTGCG-3'. The EcoRI and XmaI recognition sites are underlined.